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Global transcriptional response of porcine mesenteric lymph nodes to *Salmonella enterica* serovar Typhimurium

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Abstract

To elucidate the host transcriptional response to *Salmonella enterica* serovar Typhimurium, Affymetrix porcine GeneChip analysis of pig mesenteric lymph nodes was used to identify 848 genes showing differential expression across different times after inoculation or when compared to non-inoculated controls. Annotation analyses showed that a high proportion of these differentially expressed (DE) genes are involved in immune and inflammatory responses. T helper 1, innate/inflammatory, and antigen-processing pathways were induced at 24 h post-inoculation (hpi) and/or 48 hpi, while apoptosis and antigen presentation/dendritic cell function pathways were downregulated at 8 hpi. Cluster analyses revealed that most DE genes annotated as NFκB targets were grouped into a specific induced subcluster, while many translation-related DE genes were found in a repressed subcluster. Quantitative polymerase chain reaction analyses confirmed the Affymetrix results, revealing transcriptional induction of NFκB target genes at 24 hpi and suppression of the NFκB pathway from 24 to 48 hpi. We propose that such NFκB suppression in antigen-presenting cells may be the mechanism by which *S.* Typhimurium eludes a strong inflammatory response to establish a carrier status in pigs.

Keywords: Porcine; Gene expression; *Salmonella* typhimurium; Immune response; NFκB

Salmonella spp. are among the major causes of bacterial foodborne zoonotic infections [1], eliciting a variety of diseases, ranging from localized gastroenteritis to a life-threatening systemic disease. One *Salmonella enterica* host-generalist serovar, Typhimurium, is a gram-negative, facultative intracellular bacterium that has the potential to infect almost all vertebrates including humans, while *S.* Choleraesuis is host adapted to pigs. *S.* Typhimurium and *S.* Choleraesuis are etiologic agents of swine salmonellosis, which causes about $100 million in annual pig production losses nationwide [2]. *S.* Choleraesuis-infected pigs cause a systemic disease while *S.* Typhimurium infection in pigs leads to a localized enterocolitis and may establish a carrier state [3]. In addition, serovar Typhimurium isolated from pigs has been shown to harbor multidrug resistance [4].

S. Typhimurium-carrier animals are major threats to food safety because of the subclinical nature of the infection. Human gastrointestinal salmonellosis is caused by consumption of contaminated meat, raw milk, or eggs [5,6]. Consumption of pork is responsible for 14.6% of all known causes of foodborne illness outbreaks in the United States [7]. There are about 1.4 million cases of human nontyphoid salmonellosis in the U.S. and about 600 *Salmonella*-associated deaths occur each year [6].

S. Typhimurium infection in mice causes a systemic disease similar to human typhoid fever; thus, a murine infection model has been extensively exploited to study systemic *Salmonella* infection in humans [8]. However, *S.* Typhimurium infection in pigs

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usually causes enterocolitis which is similar to gastroenteritis in humans. On this basis, swine are regarded a good model for investigating enteric salmonellosis in humans [8,9].

Mesenteric lymph nodes (MLN) are the largest lymph nodes in humans and other animals and play an important role in immune defense against bacterial pathogens as one of the components of gut-associated lymphoid tissues (GALT). Recently, host gene expression change profiles in response to *S.* Choleraesuis infection have been conducted in pigs not only in the lung [10], the mucosa [11], and the ileum and jejunal epithelial cells [12] but also in the GALT: mesenteric lymph nodes [13] and Peyer's patch [14]. Substantial research has also been performed on the host gene response to *S.* Typhimurium in different species and cell lines [8,15–17]. However, genomewide approaches to study the porcine MLN immune response to *S.* Typhimurium infection and to identify the ways in which these bacteria attempt to thwart this response have not been reported.

As *S.* Typhimurium is a major food safety problem, and its infection in pigs is a model of choice for human gastrointestinal research, we investigated host immune response to *S.* Typhimurium in the pig. A first-generation Affymetrix GeneChip Porcine genome array, which contains oligonucleotide probesets representing approximately 23,256 transcripts from 20,201 *Sus scrofa* genes, was used to profile the gene expression in porcine mesenteric lymph nodes over a time course of infection with *S.* Typhimurium, including the acute (8 h post-inoculation (hpi), 24 hpi, 48 hpi) and chronic (21 days postinoculation (dpi)) stages of infection. The acute stage of infection was defined by the clinical manifestations and the increase in serum IFNG and TNFα levels from infected pigs occurring up to 7 days post-infection [18]. Our objectives were to (1) identify and examine the stereotypical gene expression response within host MLN to *S.* Typhimurium infection, (2) characterize global host responses by revealing the specific features of the host's innate immunity pathways, and (3) explore whether and how *S.* Typhimurium may escape the host immune response and develop into a carrier state. These studies should expand elucidation of the host–pathogen interaction globally and provide additional characterization of a valuable biological model for human nontyphoid salmonellosis.

Results

Transcriptome analysis

The transcriptome of non-infected pig MLN was determined and 14,711 probesets detected expression in this tissue. The total number of genes expressed in infected MLN for at least one time point during *S.* Typhimurium infection was also calculated. Expression was detected for 16,123 transcripts (70% of all probesets) in MLN during infection, and a total of 16,229 transcripts was expressed in infected and non-infected porcine MLN (Supplementary Table 1). To elucidate the biological processes in which these genes are involved, gene ontology (GO) annotation was performed with the 16,229 transcripts using our own GO-slim which was built with the most relevant terms for immune response in the biological process category of the GO database (Fig. 1). The results revealed that greater than 5000 probesets were assigned specific GO terms. A significant number of these genes were thus annotated as being involved in cellular metabolism, signal transduction, development, cell differentiation, and cell motility; additional genes were assigned GO terms related to immune response, cell migration, and cell adhesion, cell proliferation, and inflammatory response.

Differentially expressed (DE) gene analysis during infection

To elucidate the global transcriptional response during infection, pairwise comparisons between all 10 time points during infection were calculated. Results showed that 848 genes had p values <0.01 and fold change estimates (fc)>2 ($q<0.24$) in at least one of the pairwise comparisons (listed in Supplementary Table 2). Of these DE genes, 520 transcripts were matched to human Refseq entries by BLAST sequence similarity analyses, while the other 38% of transcripts were non-annotated. The numbers of genes declared as differentially expressed genes at each time point compared to non-infected animals (8 h-C, 24 h-C, 48 h-C, 21 day-C) are shown in Fig. 2.

GO annotation mapping of the 848 genes revealed that, in comparison to the global transcriptome GO term assignment, the proportion of genes in the 848 DE gene list which were assigned GO terms associated with cellular metabolism, development, and cell motility was slightly decreased (Fig. 1). On the other hand, the genes assigned GO terms for immune response, innate immune response, inflammatory response, and defense response were significantly ($p<0.05$) enriched in our 848 differentially expressed genes (Supplementary Table 2).

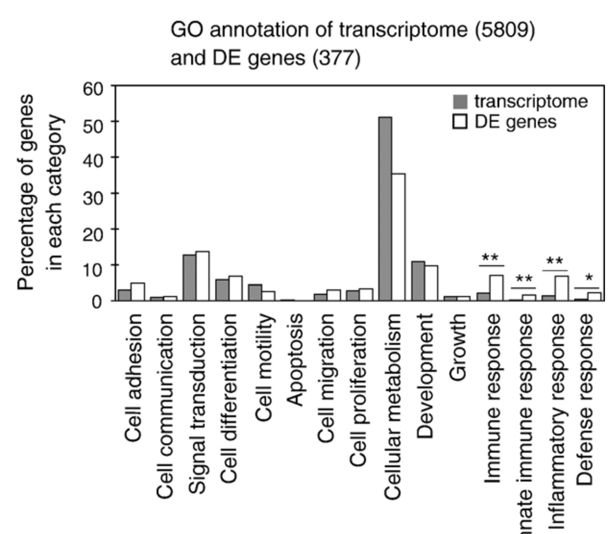

Fig. 1. Biological process gene ontology (GO) categorization of the porcine MLN transcriptome and detected 848 DE genes ($p<0.01$, fc>2, $q<0.24$). All 16,229 transcripts which were expressed in infected and noninfected porcine MLN and 848 detected DE genes were annotated using our specific GO-slim. Statistical significance of $p<0.05$ and $p<0.01$ are denoted with an asterisk (* and **). The x-axis represents each GO category and the y-axis shows the gene percentage of each GO category with regard to the MLN transcriptome or the declared DE genes.

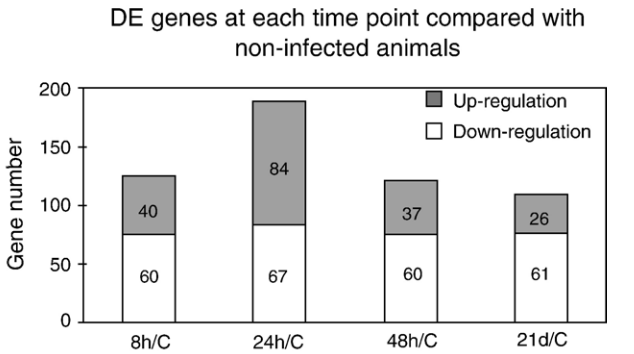

Fig. 2. Differential gene expression in the MLN of swine during *S.* Typhimurium infection. The number of declared DE genes at each time point of 8 hpi, 24 hpi, 48 hpi, and 21 dpi, compared to noninfected animals is shown ($p<0.01$, fc>2 and $q<0.24$).

The proportion of genes with other biological processes/functions assigned to them, such as cell adhesion and cell migration, was also increased during the infection, but these increases were not statistically significant (Fig. 1).

Pathway analysis

During bacterial infection, T lymphocytes will eventually encounter antigens that are carried from sites of infection to the lymph nodes by antigen-presenting cells, primarily macrophages and dendritic cells. Extensive cell migration into or out of the lymph node could cause changes in the RNA profile of the MLN, and these RNA changes might be erroneously interpreted as an RNA expression response to infection within resident tissue cells. To assess whether the observed differences in gene expression during infection are due to cellular migration, the expression levels of cell-type markers for T cells, macrophages, dendritic cells, and granulocytes, whose expression levels are not expected to change during infection, were analyzed (Fig. 3A). No significant changes in RNA levels for these marker genes were detected, and thus no evidence of significant cell migration was found. These data suggest that the majority of the RNA abundance differences observed were not due to significant changes in the abundance of specific cell types in the MLN but are likely representative of specific transcriptional or post-transcriptional responses within cells.

To further investigate the immune-related pathways activated during infection, the expression pattern of specific genes known to be involved in specific immune pathways was analyzed. The expression patterns of these genes are displayed in Fig. 3B and serve as markers for the immune-related pathways, as discussed in the following.

Th1 and Th2

Results showed that the known Th1-related genes IFNG, IRF1, SOCS1, STAT1, and WARS were significantly upregulated at 24 and/or 48 hpi (Fig. 3B), while Th2-related genes, IL4 and IL13, were downregulated or unchanged respectively at all time points. These data suggested that *S.* Typhimurium elicited primarily a Th1-associated response within the MLN during infection.

Innate immunity/inflammation/apoptosis pathways

Genes known to be involved in innate/inflammatory/apoptosis pathways, such as IL8, IL6, SLC11A1, IL1B, TGM1, and TGM2, were upregulated to different extents at 24 and/or 48 hpi, although the IL6 and IL8 responses were not statistically significant. Some genes in these pathways, such as innate/inflammatory (IL8, TLR4, IL6) and apoptosis (CASP1, CASP4, GZMB), were found to be downregulated at 8 hpi and/or 21 dpi during infection. All responses except those for IL8 were statistically significant.

Antigen processing and presentation pathways

Two antigen-processing related genes, PSMB8 and PSMB10, did not change their expression levels significantly. However, TAP1, which is known to be involved in antigen processing, was significantly upregulated at both 24 and 48 hpi. Another gene from the same gene family, TAP2, was induced at 48 hpi but did not reach a statistically significant level above the negative control.

It is interesting that several markers which are known to be involved in activation of the antigen presentation pathways (CD80 and CD86) were downregulated significantly at 8 hpi. Another gene involved in antigen presentation, CD209, which is also named DC-SIGN and expressed almost exclusively in dendritic cells, showed significant downregulation at 48 hpi and 21 dpi.

Hierarchical cluster analysis

To define sets of genes with a specific response to *S.* Typhimurium, we used hierarchical clustering to construct a heat map based on the expression pattern of the 848 genes that were declared to be differentially expressed ($p<0.01$, fc>2, $q<0.24$) (Fig. 4). At the highest level, these genes could be grouped into two distinct clusters: an induced gene cluster and a repressed gene cluster. Both clusters could be further refined into several subclusters, which are represented by bar graphs based on the centroid values of the gene cluster for better visual representation (Fig. 4). Genes that make up each subcluster can be found in Supplementary Table 2.

Eighty-six genes, including some important immune-related genes, such as IL10, PTGS1, CXCL2, CD163, GZMB, and TREM1, represent the first subcluster of the induced cluster. Genes in this subcluster were significantly downregulated at 8 hpi but raised their expression at 24 and 48 hpi to levels comparable to those of the non-infected pigs (Fig. 4, subcluster 1) before decreasing expression by 21 dpi. The expression pattern of the second subcluster, which included SCARB2, SAA1, CCR5, and CIDEB, was similar to that of the first subcluster, except that genes were significantly induced at 48 hpi (Fig. 4, subcluster 2). One specific subcluster of 129 induced genes (subcluster 3) showed a significant and strong induction from 8 to 24 hpi and a downregulation from 24 to 48 hpi. Most genes in this subcluster are annotated as NFκB target genes [19], (also see http://www.nf-kb.org), cytokines and chemokines, and INFG-induced genes, indicating that the mRNA response of these important pathways in the host

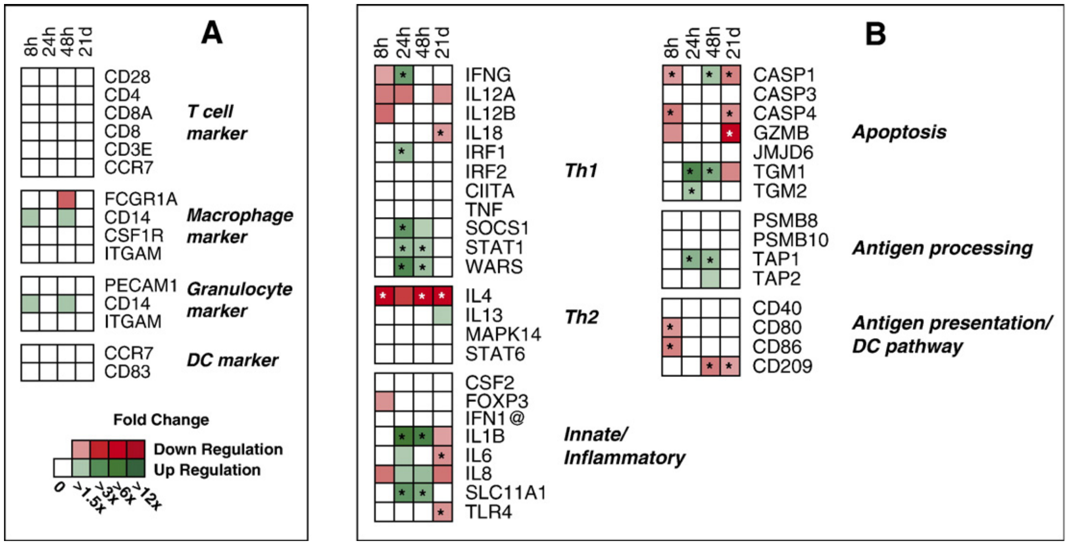

Fig. 3. Transcriptional profiles of selected cell-type marker and immune response pathway genes. Expression patterns of specific marker genes for T cell, macrophage, granulocyte, and dendritic cells (A) or genes in pathways (B) that respond to *S.* Typhimurium infection in pigs are shown. The fold change from comparisons of infected pigs and noninfected controls at each time point were calculated from the Affymetrix array data using Genecluster. Statistical differences ($p<0.05$) between control and infected pigs are represented by an asterisk (*).

occurred at 24 hpi. Genes in subcluster 4 were upregulated at both 8 and 24 hpi but returned to the levels of the non-infected controls by 48 hpi and 21 dpi. This subcluster included some NFκB-related genes, such as CEBPD, EDN1, and CCL2. The last induced subcluster included 54 genes that were upregulated at both the acute and the chronic stages of infection compared to genes of non-infected animals. This subcluster included 22 annotated genes, such as FGF2, FBXO44, and NAV2 (Fig. 4, subcluster 5).

Because many NFκB targets and immune-related genes were identified in subcluster 3, additional GO annotation of these genes was performed. Of 129 genes in subcluster 3, 80 genes had significant sequence similarity to human Refseq entries, based on our BLAST results (Table 1). We found that 22 genes

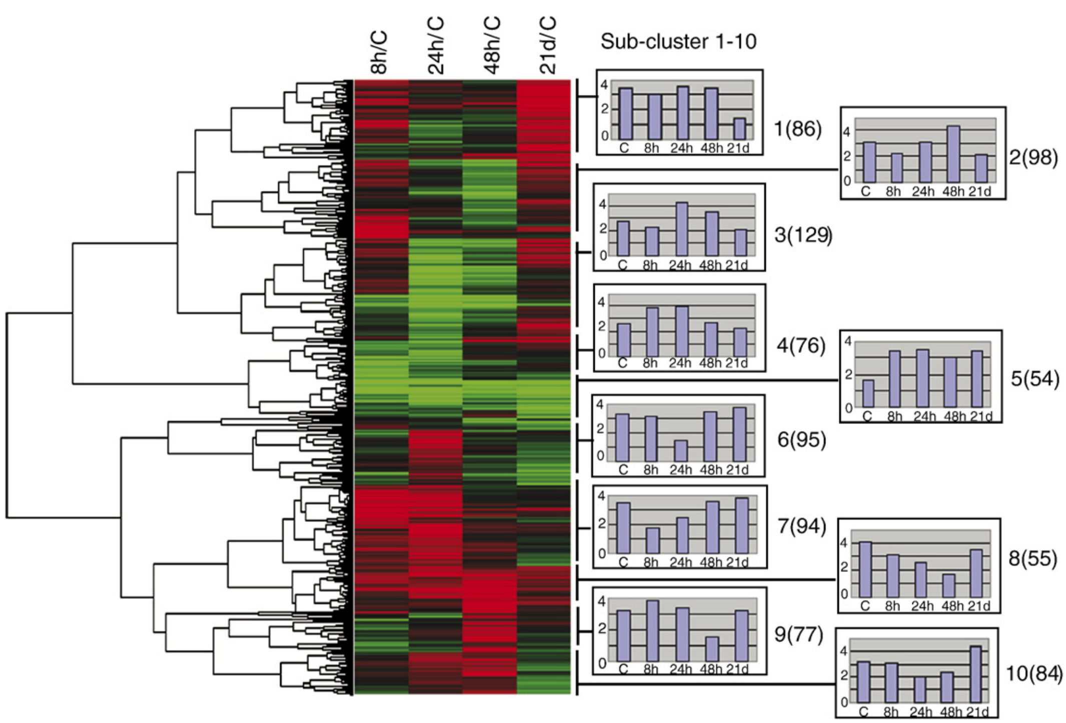

Fig. 4. Hierarchical clustering analysis of 848 declared DE genes during *S.* Typhimurium infection. The heat map was built using Gene Cluster 3.0 software and the detailed subclusters 1–10 were constructed in Gene Cluster 2.0 using centroid values. Red represents downregulation and green shows upregulation for differentially expressed genes ($p<0.01$, fc>2, $q<0.24$). The *x*-axis of each subcluster is the time point and the *y*-axis represents the centroids value. The number of genes that make up each subcluster is listed in parentheses.

were known NFκB targets and an additional 19 genes had GO annotation that indicated involvement in immune response and infection. Twenty-two genes in this subcluster had diverse GO annotations, such as binding, metabolism, and protein transport, while 8 genes with human RefSeq similarity did not have any GO annotation (Table 1).

The repressed gene cluster also had several subclusters (Fig. 4). Ninety-five genes, including 2 G-protein-related genes (SRGAP3 and RGS5), C3, and MAP3K1, had a large decrease in expression from 8 to 24 hpi, returning to the levels of the noninfected pigs by 48 hpi (Fig. 4, subcluster 6). One subcluster of 94 genes (subcluster 7) had decreased expression levels at 8 and 24 hpi, after which RNA levels returned to those seen in the noninfected animals by 48 hpi. This subcluster included many ribosomal protein genes, eukaryotic translation initiation factor 5A (EIF5A), and elongation factor 1 alpha 1 (EEF1A1). An additional subcluster of 55 genes (subcluster 8) had a lower expression pattern at the acute stages of infection compared to noninfected animals. Genes in subcluster 9 were downregulated significantly from 24 to 48 hpi. A final subcluster of 84 genes (Fig. 4, subcluster 10) represented genes that were downregulated at both 24 and 48 hpi and then upregulated from 48 hpi to 21 dpi. Subclusters 8, 9, and 10 were a diverse set of known genes, without an obvious overrepresentation of any pathway.

Additional annotation was performed for all genes in the induced (subclusters 1–5) and repressed (subclusters 6–10) clusters using the GO term mapping method that we created. Fig. 5 shows that genes which have the GO terms of cellular metabolism, immune response, and inflammatory response were overrepresented in the induced cluster compared to the repressed cluster (Fig. 5).

NFκB signaling pathway target genes investigation

Common regulators of genes in induced and repressed clusters were further examined by using Pathway Studio software (Ariadne Genomics Co., Rockville, MD). Using the GenBank accession number of the human ortholog for the DE genes and text-mining analysis, common regulators of genes in the induced and repressed clusters were identified based on knowledge about molecular interactions reported in the scientific literature. Results showed that the NFκB complex, as a common regulator, was connected directly to 28 genes from the induced cluster and to 4 genes (CTGF, MAP3K1, CAV1, IGF1) from the repressed cluster (Fig. 6). Of these 32 genes, 17 (JUNB, SELP, IL8, IL6, IL1A, IL1B, PTGS2, IFNG, SOD2, STAT1, IRF1, CXCL2, CCR5, IL10, CCR5, CCL2, and SAA1) have been identified as NFkB target genes (http://bioinfo.lifl.fr/NF-KB/) and 9 genes (JUNB, IL1B, IRF1, SOD2, IFNG, STAT1, IL6, PTGS2, and SELP) are present in subcluster 3 as shown in Table 1.

Q-PCR analysis of differentially expressed porcine genes

To confirm the differentially expressed genes declared from our microarray analysis and to focus on the apparent suppression of NFκB pathways from 24 to 48 hpi by *S.* Typhimurium, a panel of 22 genes was selected for real-time PCR analysis and validation for early response expression (8, 24, and 48 hpi). These genes included 18 known NFκB target genes (IL1A, IL15, CCL2, CCL3, CXCL5, PPBP, GBP1, GBP2, PTX3, NFKBIA, JUNB, NFKBIZ, CD14, ICAM1, TLR2, GZMB, TAP1, and CCR5), one T cell marker gene (CD4), and one macrophage marker gene CD163. In addition, although we did not find any oligonucleotide set representing TGM3 gene on the Affymetrix microarray, Q-PCR was conducted for this gene because previous research showed that it was strongly induced in lung by *S.* Choleraesuis infection [10]. TREM1 was also selected for Q-PCR validation because our microarray data showed that its expression pattern is similar to those of known NFκB target genes, although no reports have shown that TREM1 is an NFκB-regulated gene.

Real-time PCR results are in Table 2 and Fig. 7. Comparison of the Q-PCR results with the microarray data demonstrated that 19 of 21 genes had statistically significant expression patterns which were similar to those seen in the microarray data, indicating that our microarray data are highly reliable and accurate. The Q-PCR results for TGM3 showed a peak response at 24 hpi with a fold change of 69 and had an expression pattern similar to those of TGM1 and TGM2 in the MLN during infection (Fig. 3B).

Discussion

Although systematic analysis of the porcine transcriptional response to infection with various pathogenic microorganisms using microarray technology [10,14,20–22] or large-scale quantitative PCR methods [23] has been reported, this study is the first to report data using the Affymetrix GeneChip Porcine Genome Array and Q-PCR to investigate the transcriptional response to *S.* Typhimurium infection. Our results showed that the MLN transcriptomes from non-infected and infected pigs were significantly interrogated by this approach, as expression of more than 16,000 transcripts was reproducibly detected in this analysis. The GO consortium provides a defined vocabulary of gene functions in cells [24], and GO terms are now widely accepted as a useful means to annotate gene array elements. To elucidate the biological processes in which these transcripts are involved, a reduced GO vocabulary (GO-slim) that concentrates on host immune response was established and used for GO annotation analysis. Results showed that more than 1/3 of the transcriptome (>5000 probesets) were annotated using the specific GO terms that we selected. Thus we believe that the expressed transcripts in this study represent a high proportion of the porcine genomic response to *Salmonella* infection within MLN and that the GeneChip Porcine Genome Array is a very powerful tool to detect host transcriptional defense against bacterial pathogens.

Statistical analysis of differential expression revealed 848 genes with altered expression levels across one or more of the 10 possible pairwise comparisons during infection. Many annotated genes were found to overlap with those that have been implicated in the host response to infection [25] and are discussed below. GO annotations were also determined for these genes. Compared to the transcriptome GO term totals, as expec-

Table 1
Further annotation of 80 genes with human sequence similarity that clustered together based on expression pattern at 24 and 48 h postinfection

Affy ID	Gene	8 h/C fc	8/C *p*	24/C fc	24/C *p*	48/C fc	48/C *p*	Additional information
Ssc.10881.1.S1_at	JUNB	−1.301	0.259	1.308	0.250	1.161	0.513	Known NFkB target genes
Ssc.12446.1.A1_at	CASP4	−2.033	0.006	1.440	0.106	1.350	0.175	
Ssc.16250.1.S2_at	IL1RN	−1.651	0.291	4.910	0.005	2.643	0.056	
Ssc.17573.1.S1_at	IL1B	−1.127	0.762	3.949	0.005	2.741	0.026	
Ssc.19494.1.S1_at	IRF1	−1.148	0.382	2.066	0.001	1.229	0.202	
Ssc.21162.1.S1_s_at	IRF7	−1.287	0.333	2.385	0.006	1.291	0.328	
Ssc.221.1.S1_at	MX1	−1.482	0.181	1.682	0.087	1.512	0.161	
Ssc.2381.1.A1_at	S100A9	−2.779	0.051	13.113	0.000	6.338	0.003	
Ssc.27433.1.S1_at	TGM1	1.367	0.396	3.486	0.005	2.496	0.027	
Ssc.27863.1.S1_at	TAP1	1.009	0.968	2.263	0.004	1.968	0.011	
Ssc.29054.3.S1_at	GBP1	−1.452	0.069	2.234	0.001	1.748	0.012	
Ssc.3706.1.S2_at	SOD2	−1.143	0.636	2.832	0.003	2.045	0.026	
Ssc.4093.1.A1_at	IFNG	−1.517	0.092	2.780	0.001	1.207	0.419	
Ssc.6025.1.S1_at	STAT1	−1.162	0.200	2.102	0.000	1.699	0.001	
Ssc.62.2.S1_a_at	IL6	−1.064	0.803	1.624	0.072	−1.032	0.898	
Ssc.719.1.S1_at	CXCL5	−1.522	0.349	2.877	0.033	1.567	0.319	
Ssc.7314.1.A1_at	PTGS2	−1.117	0.737	2.110	0.042	1.440	0.281	
Ssc.8162.1.S1_at	PTX3	−1.131	0.790	10.963	0.000	1.479	0.407	
Ssc.883.1.S1_a_at	GBP2	−1.417	0.084	2.579	0.000	1.661	0.019	
Ssc.8833.1.S1_at	IL15	−1.402	0.005	1.876	0.000	1.340	0.011	
Ssc.9117.1.S1_at	S100A12	3.765	0.029	8.789	0.002	8.724	0.002	
Ssc.290.1.S1_at	SELP	−1.258	0.312	1.509	0.085	−1.095	0.682	
Ssc.17100.1.S1_at	S100A8	1.307	0.411	6.603	0.000	3.592	0.002	Genes with following GO terms: immune response, inflammatory response, defense response, cytokine activity, antimicrobial humoral response, or response to stimulus
Ssc.7864.1.A1_at	IL1RAP	−1.750	0.114	3.630	0.003	1.659	0.148	
Ssc.9738.1.A1_at	CEBPB	−1.034	0.899	2.186	0.012	1.811	0.043	
Ssc.30752.1.S1_at	IFIT1	−2.267	0.015	1.350	0.305	1.122	0.688	
Ssc.30887.1.S1_at	TNFAIP6	2.231	0.350	12.336	0.012	6.784	0.041	
Ssc.31140.1.S1_at	IFIT3	−1.777	0.033	1.862	0.024	1.471	0.129	
Ssc.37.1.S1_at	HP	−2.236	0.312	5.972	0.040	−2.781	0.206	
Ssc.300.1.S1_at	SLC11A1	1.083	0.626	3.057	0.000	2.444	0.000	
Ssc.27574.1.S1_at	LTBR	1.291	0.086	1.779	0.002	1.462	0.018	
Ssc.11098.1.S1_at	IFITM3	−1.858	0.025	1.200	0.458	1.137	0.598	
Ssc.11557.1.A1_at	ISG15	−1.610	0.227	2.068	0.078	1.853	0.127	
Ssc.12781.1.A2_at	TLR4	−1.280	0.135	1.414	0.045	1.072	0.658	
Ssc.12918.2.A1_at	NMI	−1.108	0.581	1.546	0.035	1.206	0.320	
Ssc.12197.1.S1_at	CMTM6	−1.336	0.203	1.538	0.070	1.280	0.273	
Ssc.22620.1.S1_at	IFIT2	−1.749	0.184	2.423	0.047	1.807	0.162	
Ssc.15885.1.S1_at	DDX58	−1.209	0.485	2.048	0.021	1.277	0.373	
Ssc.21582.1.S1_at	UBD	−1.471	0.014	1.422	0.022	1.389	0.030	
Ssc.26216.2.A1_at	SOCS1	1.065	0.761	2.356	0.002	1.235	0.321	
Ssc.24732.1.S1_at	BBS5	−1.244	0.082	1.340	0.027	1.051	0.670	
Ssc.1024.1.S1_at	SNTB1	1.116	0.645	2.101	0.009	1.425	0.157	Both genes had GO term: cytoskeleton
Ssc.18261.1.S1_at	PSTPIP2	−1.452	0.077	2.437	0.001	1.933	0.006	
Ssc.11244.1.A1_at	MBD5	−6.323	0.001	1.205	0.669	−1.134	0.772	Genes with GO term annotations covering diverse biological functions, such as binding, metabolism, protein transport and others
Ssc.13128.1.A1_at	FLJ20035	−1.192	0.426	1.973	0.009	1.522	0.076	
Ssc.13226.1.A1_at	PARP9	−1.398	0.049	1.446	0.034	1.113	0.491	
Ssc.1332.1.S1_at	SULT2A1	−1.277	0.567	2.949	0.026	2.295	0.072	
Ssc.13992.1.A1_at	KLF5	1.505	0.028	2.239	0.000	2.066	0.001	
Ssc.21663.1.A1_at	LIPG	−1.016	0.961	2.693	0.011	1.477	0.247	
Ssc.2387.1.S1_at	GNG10	−1.561	0.000	1.205	0.014	1.065	0.340	
Ssc.26326.1.S1_at	CYP3A7	−2.002	0.305	4.167	0.051	1.885	0.347	
Ssc.27381.1.A1_at	SEMA3A	−1.611	0.069	1.316	0.268	1.225	0.406	
Ssc.28312.1.A1_at	TCF7L2	1.577	0.032	2.373	0.001	1.954	0.004	
Ssc.28913.1.A1_at	GNB4	−1.398	0.111	1.538	0.049	−1.029	0.884	
Ssc.30724.1.S1_at	HERC6	−1.269	0.347	2.026	0.015	1.307	0.293	
Ssc.4989.1.A1_at	CTH	−1.708	0.012	1.202	0.321	−1.538	0.035	
Ssc.5119.1.S1_at	SLC25A28	−1.096	0.529	1.644	0.005	1.222	0.185	
Ssc.5127.1.S1_at	FBP1	−1.312	0.133	1.484	0.039	1.198	0.302	
Ssc.5663.1.S1_at	VCAN	−1.440	0.212	1.747	0.069	1.142	0.638	
Ssc.6139.1.S1_a_at	WARS	1.146	0.530	3.498	0.000	1.935	0.010	

(continued on next page)

Table 1 (*continued*)

Affy ID	Gene	8 h/C fc	8/C *p*	24/C fc	24/C *p*	48/C fc	48/C *p*	Additional information
Ssc.6191.1.S1_at	DTX3L	−1.264	0.139	1.868	0.002	1.187	0.267	
SscAffx.1.1.S1_at	ISG20	−1.451	0.157	2.588	0.003	1.755	0.043	
Ssc.6797.1.S1_at	STXBP1	−1.037	0.830	2.692	0.000	1.967	0.002	
Ssc.7275.2.A1_at	KYNU	−1.463	0.032	1.431	0.041	1.237	0.194	
Ssc.7713.1.A1_at	LAP3	−1.151	0.347	2.114	0.000	1.581	0.009	
Ssc.9693.1.A1_at	TMEM100	−1.241	0.371	1.454	0.135	−1.388	0.185	Genes without GO term annotation
Ssc.9726.1.A1_at	DRAM	−1.249	0.077	1.810	0.000	1.668	0.001	
Ssc.17005.1.A1_at	ARRDC4	−1.361	0.126	1.586	0.032	1.231	0.286	
Ssc.19365.2.S1_at	FAM26F	1.009	0.962	2.762	0.000	1.849	0.009	
Ssc.25996.1.A1_at	IBRDC3	−1.252	0.333	1.777	0.026	1.489	0.101	
Ssc.19389.1.A1_at	C15orf48	1.201	0.292	2.271	0.001	1.426	0.056	
Ssc.6382.1.A1_at	PPP1R3B	−1.375	0.125	1.254	0.261	−1.128	0.541	
Ssc.30474.1.A1_at	C15orf26	−3.994	0.055	1.971	0.312	1.128	0.854	
Ssc.11170.1.S1_at	PDXK	−1.096	0.578	1.454	0.040	1.519	0.025	Genes that were up-regulated slightly from 24 to 48 hpi, even they were grouped into subcluster 3
Ssc.11583.1.A1_at	SLC2A6	1.216	0.541	2.124	0.035	2.408	0.018	
Ssc.15593.2.S1_at	WFS1	1.433	0.072	2.175	0.001	2.204	0.001	
Ssc.18359.1.S1_at	CCR1	−2.085	0.004	1.501	0.070	1.604	0.040	
Ssc.26146.1.S1_at	CXCL9	−1.410	0.214	2.739	0.003	3.243	0.001	
Ssc.27201.1.S1_a_at	CCRL2	−1.779	0.001	1.763	0.001	1.953	0.000	
Ssc.658.1.S1_at	IL8	-2.135	0.057	1.786	0.131	2.021	0.074	

ted, the number of genes among our 848 DE gene list that were annotated as being involved in immune response, innate immune response, and inflammatory response pathways was dramatically increased (Fig. 1).

These data may be useful in finding novel genes controlling immune response in the pig and other mammals, including human. Further, the DE genes identified in this study, both genes with known immune function and those with unknown function, are useful candidate genes for investigating the association between immune-related traits and genetic variation. Polymorphisms at these candidates might be valuable markers for enhancing disease resistance, pig health, and food safety by molecular breeding methods.

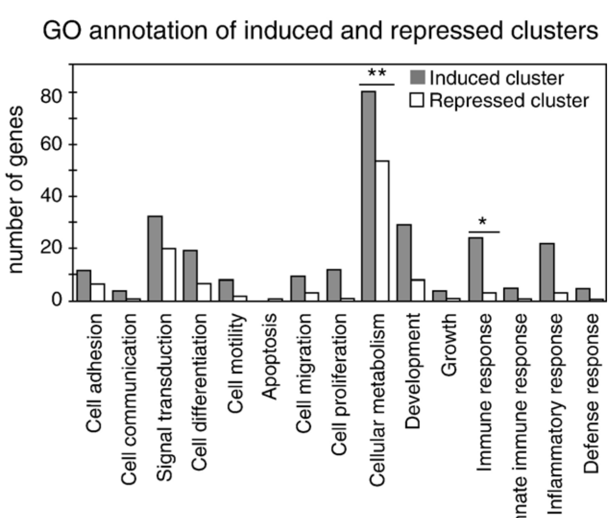

Fig. 5. Biological process gene ontology (GO) categorization of declared DE genes in induced cluster (subclusters 1–5) and repressed cluster (subclusters 6–10). Statistical significance is denoted with an asterisk (*$p<0.05$ and **$p<0.01$). The x-axis represents each GO categories and the y-axis is the gene number of each GO category.

Pathway analysis

Infection of swine with *S.* Typhimurium elicited a Th1-type response at early time points, as shown by IFNG stimulation and by induction of some IFNG-signaling-responsive genes, such as SOCS1, STAT1, WARS, and IRF1 (Fig. 3B); this is consistent with the results from *Salmonella* infection studies in mouse [26] and *S.* Choleraesuis infection in pigs [10] (Y. Wang et al., unpublished data). However, of interest is that both IL12A and IL12B were downregulated in our study (Fig. 3B). A low expression level of IL12 has been reported in the porcine response to reproductive and respiratory syndrome virus [27] and it has been observed in a Q-PCR analysis in porcine MLN during infection (J.J. Uthe et al., unpublished). The function of IL12 during *Salmonella* infection appears to be complex [8]; moreover, pig IL12 only weakly stimulates swine cells, with poor upregulation of IL12R [28]. IL12, which has been shown to have IFNG-inducing properties [29], has also been described as having a role in maintaining rather than inducing IFNG by T cells during *Salmonella* infection [30]. In addition, IL18, another gene that is thought to have IFNG-inducing properties, was downregulated at 21 dpi and IL18 expression was not changed at other time points. Suppression of IL18 expression was also observed in *Salmonella*-activated murine macrophages and in *Salmonella*-infected mice [31,32]. Therefore, we predict that lack of activation, or even suppression of, IL12 and IL18 expression during *S.* Typhimurium infection might stifle IFNG induction and affect the host defense against *Salmonella*. The IL4 gene, which initiates the primary Th2 response, was downregulated significantly in porcine

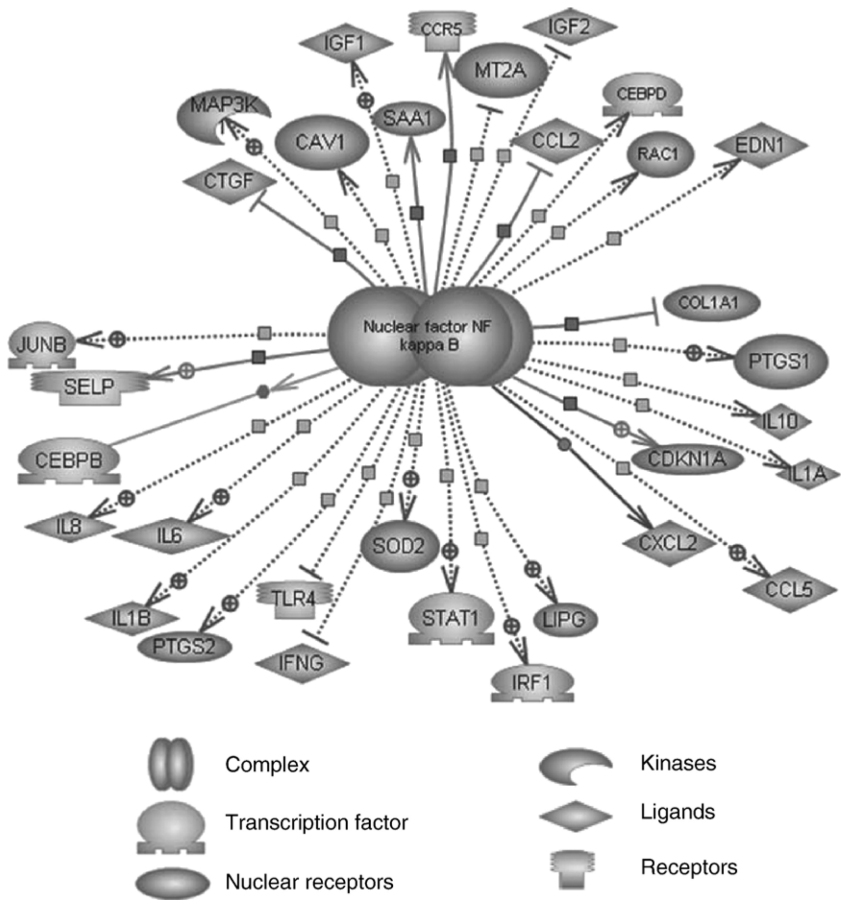

Fig. 6. Pathway Studio software illustrates that 32 genes identified as differentially expressed in response to *S.* Typhimurium infection are targets of the NFκB complex. The NFκB pathway diagram was built by using the ResNet curation of the PathwayStudio software.

MLN during infection, and IL13, which also predominately drives a porcine Th2 response, did not change its expression level significantly. These results indicate that the Th2 response was suppressed at the early stages of infection.

Fig. 3B shows that several genes which are involved in innate immunity/inflammatory (IL6, IL8, IL1B) and apoptosis (CASP1 and TGM1) pathways, displayed specific expression patterns; they were induced in response to *S.* Typhimurium infection at 24 and/or 48 hpi but not changed or downregulated at 8 hpi and 21 dpi. Of note is the SLC11A1 gene, also named NRAMP1, which has been reported to play an important role in controlling the replication of intracellular bacteria and resisting *Salmonella* infection in mouse and chicken [33]. SLC11A1 was upregulated significantly at 24 and 48 hpi in porcine MLN, consistent with the results from *S.* Choleraesuis infection in pig lung [10], and induction of SLC11A1 expression in porcine MLN during *S.* Typhimurium infection has recently been confirmed by Q-PCR analysis [18]. TLR4, the early lipopolysaccharide (LPS) sensor responding to a broad range of microbes, did not show a dramatic change in expression during infection. Its expression level was elevated 1.4-fold ($p = 0.045$) at 24 hpi but was downregulated significantly at 21 dpi. Significantly increased expression of TLR4 at 48 hpi was observed in porcine lung and MLN during *S.* Choleraesuis infection [10] (Y. Wang et al., unpublished data). Our data suggested that weak and transient induction of TLR4 might be one of the reasons that the pig host lacks a strong inflammatory response during *S.* Typhimurium infection.

In our study, apoptosis-related genes such as CASP1, CASP3, CASP4, and GZMB were downregulated at an early stage of infection, which indicates that *S.* Typhimurium might interfere with cell death signaling, thereby increasing its chance to survive. Downregulation of proapoptotic genes in early infection was also observed in human alveolar macrophages infected with virulent *Mycobacterium* tuberculosis [34]. In addition, TGM1, TGM2, and TGM3, which are members of the transglutaminase gene family and may be involved in apoptosis [35], showed quite similar expression patterns during infection in our study: they were significantly upregulated at 24 and/or 48 hpi, while no change in expression or downregulation was observed at 8 hpi and/or 21 dpi compared to noninfected pigs. In comparison to the response of TGM3 to *S.* Choleraesuis in the porcine lung where strong induction was seen at 48 hpi [10], TGM3 reached a peak response at 24 hpi (with fold change of 69 compared to noninfected animals in Q-PCR analysis). Although the role that these transglutaminase genes play in inflammation and apoptosis is not yet clear, recent evidence demonstrates that increased TGM2 activity can trigger NFκB activation without NFKBIA kinase signaling [36].

Table 2
Q-PCR results for gene expression at each early response stage (8, 24, and 48 hpi) in *S.* Typhimurium infection

Gene name	Control			8 hpi			24 hpi			48 hpi		
	Average C_t [a]	SD	Stat [b]	Average C_t	SD	Stat	Average C_t	SD	Stat	Average C_t	SD	Stat
IL1A	27.4	1.01	A	27.4	1.3	A	24.4	2.32	B	26.1	0.36	AB
IL15	25.8	0.56	A	26.0	0.2	A	24.0	0.12	B	24.9	0.12	C
CCL2	20.7	0.38	A	20.4	0.6	AB	19.7	0.38	B	20.6	0.24	AB
CCL3	28.5	0.18	AB	28.6	0.1	B	27.9	0.83	AB	27.5	0.52	A
CXCL5	28.2	0.08	AB	29.1	0.8	B	26.7	0.53	AC	27.4	0.98	AC
PPBP	32.1	0.73	A	33.3	0.4	A	29.0	1.48	B	27.2	0.89	B
GBP1	22.4	1.18	A	22.4	0.4	A	20.0	0.69	B	21.0	0.33	B
GBP2	24.5	0.64	A	24.7	0.2	A	22.6	0.67	B	23.4	0.18	B
PTX3	27.2	0.65	A	27.1	0.5	A	23.1	1.00	B	26.0	0.15	A
NFKBIA	25.4	0.29	A	25.2	0.5	A	25.2	0.07	A	25.0	0.17	A
JUNB	30.4	0.18	A	30.7	0.6	A	30.3	0.09	A	30.4	0.18	A
NFKBIA	22.6	0.57	A	22.7	0.8	A	22.2	0.60	A	22.3	0.12	A
TAP1	22.0	0.23	AB	22.2	0.3	B	20.9	0.32	C	21.5	0.19	A
GZMB	22.2	0.13	A	21.0	1.9	A	21.1	1.56	A	21.5	0.67	A
CCR5	26.1	1.11	A	26.2	0.3	A	24.9	0.37	B	24.2	0.09	B
TLR2	24.1	0.73	A	24.4	0.5	A	23.2	0.19	B	23.4	0.25	A
CD14	27.5	0.38	A	27.5	0.8	A	26.8	0.27	A	26.9	0.34	A
ICAM1	29.0	0.41	A	28.8	0.7	A	28.3	0.10	A	28.7	0.26	A
TGM3	26.4	2.96	A	26.5	2.7	A	20.3	1.11	B	23.2	1.33	B
TREM1	25.2	0.07	AB	26.6	1.0	A	23.3	0.62	C	23.9	0.43	BC
CD163	21.9	0.69	A	22.3	0.5	A	21.6	0.38	A	21.7	0.02	A
CD4	23.3	0.71	A	22.7	0.5	A	22.8	0.10	A	22.7	0.38	A
RPL32	16.9	0.49	A	17.2	0.4	A	16.6	0.19	A	16.9	0.07	A

[a] C_t, cycle threshold: the cycle number in which amplification crosses the threshold set in the geometric portion of amplification curve, lower C_t means higher expression level.
[b] C_t values for the same gene not connected by same letter are significantly different at $p \leq 0.05$ level across different time points.

The TREM1 gene encodes a newly discovered cell surface molecule expressed on neutrophils and some monocytes [37], and its overexpression can amplify the TLR-initiated responses to bacteria [38]. It has been reported that the expression level of TREM1 in bone marrow cells derived from *S.* Typhimurium-infected pigs was upregulated at 8 and 24 hpi but dramatically downregulated at 48 hpi [39], which is not consistent with our data (Fig. 7). The difference in expression levels during *S.* Typhimurium infection in the porcine MLN and bone marrow cells could easily be due to different responses of gut tissue versus bone marrow cells to this pathogen.

Antigens must be processed into peptides before they can be presented to naïve T cells by MHC molecules on antigen-presenting cells. Two genes involved in antigen processing, TAP1 and TAP2, exhibited an increased expression level early in the infection in our study, which is consistent with the gene expression patterns observed in the porcine lung during *S.* Choleraesuis infection [10]. These data illustrate that the antigen-processing pathway was activated in response to *S.* Typhimurium. Interestingly, two cell surface molecules involved in antigen presentation, CD80 and CD86, were downregulated early in infection, and no expression differences with respect to noninfected pigs were observed at late stages. The specific dendritic cell (DC) function gene, CD209 (DC-SIGN), was downregulated significantly at 48 and 21 dpi. These data suggest that the porcine DC-mediated antigen-presentation pathway was impaired during infection, which is consistent with the conclusion from other researchers that antigen presentation by murine DC cells can be directly inhibited by *S.* Typhimurium [40,41]. Thus we predict that, as in the mouse, subversion of DC function in the pig by *S.* Typhimurium may prevent efficient stimulation of T cell proliferation, and this may be crucial for survival of the pathogen by escaping DC-mediated antigen presentation. It is interesting, however, to note that *S.* Typhimurium in the mouse is able to develop a systemic infection, presumably through early interference of DC function, while *S.* Typhimurium infection in the pig is contained within the gut and in gut-associated lymph tissue. Nevertheless, the DC evasion may play a role in the carrier status of *Salmonella* in swine, although the specific site(s) of carriage have not been clearly resolved.

Cluster analysis

The 23,256 transcripts on the porcine genome array have not been completely annotated because of limited availability of full-length porcine cDNA and because many human/mouse genes do not have functional annotation. Gene clusters created by grouping genes of similar expression patterns can help not only to annotate "unknown genes" with coexpression data to "known genes" in the same cluster but also to characterize gene network regulatory mechanisms involved in infection response. In this study, hierarchical cluster analysis was performed using the 848 genes that were differentially expressed in at least one pair of time points in the infection. Two large clusters were identified at the highest level: an induced cluster and a repressed cluster. Many genes in the induced cluster were

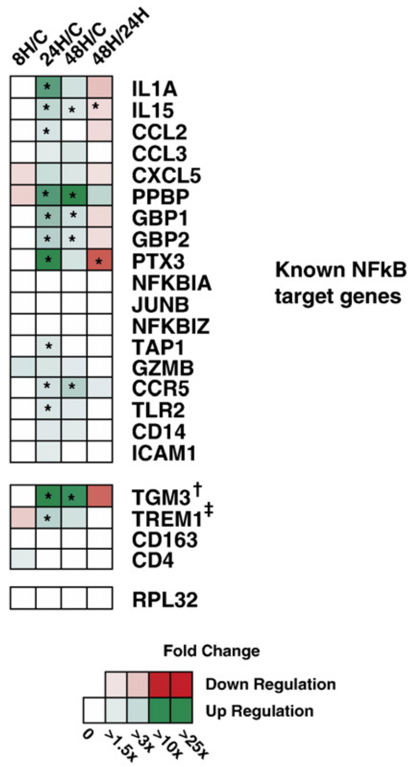

Fig. 7. Quantitative PCR analysis validates transcriptional profiling data for genes responding to *S.* Typhimurium infection. Real-time Q-PCR data are presented as the fold change in gene expression in infected pigs compared to that in the negative controls and comparing gene expression from 24 to 48 hpi. Statistical significance ($p<0.05$) is denoted with an asterisk (*). †TGM3 showed dramatic increases in porcine lung during *S.* Choleraesuis infection; thus TGM3 was also selected for Q-PCR analysis. ‡Even though TREM1 is not a known NFκB target gene, it was selected for Q-PCR analysis due to its expression pattern being similar to that of NFκB-dependent genes.

annotated with immune response and inflammatory response terms, which is consistent with the results from the pig response to *S.* Choleraesuis infection, where a higher proportion of immune-response-related genes was found in induced gene clusters than in repressed gene clusters [10]. These data indicate that induction of gene expression (rather than repression) is a main indicator of immune response during infection. One of the features of the early host response to infection that we observed is the repression at 8 and 24 hpi of some genes that are involved in ribosome assembly and maintenance or in translation initiation and elongation. This effect is similar to the response to LPS in skeletal muscle of neonatal and adult pigs [42,43], to the response to endotoxin in human blood leukocytes [44], and to the porcine MLN response to *S.* Choleraesuis (Y. Wang, et al., unpublished data), where a large number of genes involved in translation were repressed. This might be evidence that an early effect of *S.* Typhimurium on the host is suppression of translation.

Genes found in subclusters 1, 2, and 3 (Fig. 4) are clearly involved in Th1, innate/immune response, and apoptosis pathways (Fig. 5). Eighty genes in subcluster 3, with similarity to human RefSeq entries, were further analyzed by GO annotation. We found not only that many NFκB targets and immune-related genes were in this subcluster but also that metabolic- and binding-related genes were present. As these genes exhibited an expression pattern similar to that of NFκB target genes, such genes might play an important role in host response to bacterial infection. Future analysis of these genes may help to extend the knowledge of host immune response into additional cellular processes such as cell proliferation and cell metabolism, as marked by these genes.

To further analyze the genes in these subclusters, genes in the induced cluster (subclusters 1–5) were subjected to Pathway Studio literature-mining software analysis to find common regulators of these genes. Results showed that the NFκB complex can be linked to many of these genes and that this signaling pathway is centrally involved in the response to *Salmonella* infection in the pig (Fig. 6). Of these NFκB-related genes, 17 have been previously identified as NFκB direct targets (http://bioinfo.lifl.fr/NF-KB/) and 9 NFκB target genes were grouped in subcluster 3 based on their expression patterns as shown in Table 1. In addition, 19 genes with immune annotation and other genes which have not been shown to be involved in immune function were grouped in the same subcluster with known NFκB target genes due their similarity in expression patterns. Therefore, we predict that some of these genes might be NFκB target genes; experimentation to test such relationships will be needed to confirm this hypothesis.

The expression profiles for 18 NFκB target genes were further confirmed by Q-PCR analysis. We found that NFκB signaling was transiently activated from 8 to 24 hpi during *S.* Typhimurium infection but not from 24 to 48 hpi. This might result in either a lack of stimulation or a downregulation of many innate immune-related genes of the host. Known NFκB-regulated genes, which harbor the NFκB regulatory element sequence within their promoter region in other species, such as IL1A, IL15, CXCL5, CCL2, CCL3, ICAM1, and many other NFκB-dependent genes, TLR2, GZMB, and PTX3, etc., exhibited this expression pattern. Our data allow us to suggest that the rapid but transient induction of NFκB pathways in cells responding to *S.* Typhimurium infection may allow the bacteria a greater chance to survive.

What causes an early repression of the NFκB pathway in the *S.* Typhimurium-infected gut is not clear. Recently, some researchers have presented evidence that intracellular *Salmonella* are able to attenuate the host's immune response by shutting down NFκB signaling [45]. How *S.* Typhimurium interferes with activation of NFκB remains unclear, although some investigations have shown that two *S.* Typhimurium translocated leucine-rich repeat effector proteins, SspH1 and SptP, can inhibit NFκB-dependent gene expression [46]. In this context, we assessed the expression levels of NFKBIA (IkBα) and NFKBIZ (IkBz) genes during infection, as the expression of both these inhibitory genes are activated by NFκB in a negative feedback loop, which provides an effective mechanism for controlling NFκB activity [47,48]. Both our microarray and Q-PCR data showed that both genes did not change their expression level compared to noninfected pigs, indicating that NFκB activity undergoes an early and highly transient stimulation in porcine MLN during *S.* Typhimurium infection that is suppressed without demonstrable feedback inhibition.

Our study has attempted to investigate the features of host gene expression profiling during *S*. Typhimurium infection at the acute and chronic infection stages and to explore the mechanism by which *S*. Typhimurium can escape from the host immune response and develop a carrier state in the host. In conclusion, by using the Affymetrix porcine GeneChip, 848 differentially expressed genes were identified in porcine MLN during infection and several specific features of host response were revealed by gene cluster and pathway analysis. Our data are the first reported from studies to investigate global host responses to *S*. Typhimurium in porcine MLN, and this new study provides data applicable for studying enteric salmonellosis of pigs and humans.

Materials and methods

Experimental animals and tissue collection

Fifteen piglets from *Salmonella* spp.-free sows were weaned at 10 days (d) of age, shipped to the National Animal Disease Center, Ames, IA, and raised in isolation facilities. To confirm that all piglets were free of *Salmonella* spp. prior to challenge, bacteriological cultures were performed twice on rectal swabs before the experiments. At 7 weeks of age, 3 pigs were randomly allocated to the noninfected group and 12 to the infected group. The 3 noninfected control pigs were necropsied 2 days prior to experimental infection. On day 0, pigs in the infected groups were intranasally challenged with 1×10^9 colony-forming units of *S*. Typhimurium χ4232. A randomly chosen group of 3 infected pigs was necropsied at each time point of 8 hpi, 24 hpi, 48 hpi, and 21 dpi. Tissue samples from the MLN were collected and immediately frozen in liquid nitrogen. Total RNA was isolated from ~200 mg of these samples by using the RNeasy Midi kit with on-column RNase-free DNase digestion (Qiagen, Valencia, CA) based on the manufacturer's protocol. The integrity, quality, and quantity of RNA were assessed using the Agilent Bioanalyser 2100 and RNA Nano 6000 Labchip kit (Agilent Technologies, Palo Alto, CA).

Microarray hybridizations and data analysis

Five micrograms total RNA was used for first- and second-strand cDNA synthesis according to manufacturer instructions (Affymetrix, Inc., Santa Clara, CA). The double-stranded cDNA was purified and tested on an Agilent Bioanalyser 2100 and served as a template for the subsequent in vitro transcription (IVT) reaction for cRNA amplification. Labeling with cRNA biotin was performed by the GeneChip One-Cycle target labeling kit (Affymetrix; Expression Analysis Technical Manual). Quality of the labeled cRNA was tested on an Agilent Bioanalyser 2100. Subsequently, labeled cRNA was fractionated and hybridized with the GeneChip Porcine Genome Array according to the standard procedures provided by the manufacturer. Chips were washed and stained with a GeneChip Fluidics Station 450 (Affymetrix) using the standard fluidics protocol. Chips were then scanned with an Affymetrix GeneChip Scanner 3000 (Affymetrix).

MAS 5.0 (microarray analysis system 5.0, Affymetrix) default normalization methods were used to obtain the expression measure for each probeset. Logarithms were then taken on these expression measures. The median of the log expression measures for each chip was then subtracted from all the log expression measures on the same chip. Differentially expressed genes were identified by analyzing these normalized data using a general linear model in SAS (SAS Insititue, Cary, NC) on a gene by gene basis. The statistical model for gene *g* was $y_{ijg}=\mu_g+T_{ig}+\varepsilon_{ijg}$, where y_{ijg} is the log of the normalized signal for gene *g*, μ_g is an intercept term for gene *g*, T_{ig} is the fixed effect of the *ith* time point on expression of gene *g*, and the ε_{ijg} values are independently normally distributed random errors with mean 0 and gene-specific variances. An *F* test for differences in expression across all of time points during infection and *t* tests for all 10 pairwise comparisons among the five treatment groups (noninfected, 8 hpi, 24 hpi, 48 hpi, and 21 dpi) were conducted as part of the analysis for each gene. This yielded 11 sets of *p* values for the effect of infection. Each set of *p* values was converted to a set of *q* values using the method of Storey and Tibshirani [49]. The largest *q* value in a list of genes declared to be differentially expressed provides an estimate of the upper bound of the positive false discovery rate associated with the list. The microarray data have been deposited in the NCBI GEO database (Accession No. GSE7313).

Transcriptome determination

Affymetrix GeneChip porcine genome array probeset contains 11 paired perfect match (PM) and mismatch (MM) 25-mer probes, which are used to determine whether a given gene is expressed and to measure the gene expression level. The probe-pair (PM-MM) data were used to estimate the detection call (present call, marginal call, and absent call) by MAS 5.0 (Wilcoxon signed rank test). A probeset is called present when significantly more PM oligonucleotides show higher hybridization signal than their corresponding MM oligonucleotides. Transcripts which showed a present call for all three noninfected animals were counted in the transcriptome of porcine MLN tissue. Transcripts which showed a present call for all three replicates at least one time point during infection were counted as the transcriptome of infected porcine MLN tissue.

Cluster analysis

A total of 848 genes that were found to be differentially expressed ($p<0.01$, fold change >2, and $q<0.24$) in at least 1 of the 10 possible time point pairwise comparisons (8 h-C, 24 h-C, 48 h-C, 21 d-C, 24 h-8 h, 48 h-8 h, 21 d-8 h, 48 h-24 h, 21 d-24 h, and 21 d-48 h) in the *S*. Typhimurium infection were used in a hierarchical cluster analysis and to construct a heat map using the Gene Cluster 3.0 and tree view software (Stanford University, 2002). A bar graph of 10 subclusters was constructed by using centroid values obtained from Gene Cluster 2.0 analysis [50].

GO-slim creation and GO annotation of Affymetrix probesets

A set of high-level GO terms (including cell adhesion, cell communication, signal transduction, cell differentiation, cell motility, apoptosis, cell migration, cell proliferation, cellular metabolism, development, growth, immune response, innate immune response, inflammatory response, and defense response) which represent the host response categories in biological process was selected by using OBO-Edit, which is part of the go-dev software provided by GO at Sourceforge (https://sourceforge.net/project/showfiles.php%3Fgroup_id%3D36855%26package_id%3D33201). Using the selected GO terms as input for go-show-paths-to-root.pl from go-dev, all the pathways from the desired GO terms to the root of the DAG-all were used to create a valid OBO file using the original GO OBO flat file.

To assign the GO terms to the probesets on the Affymetrix array, the Affymetrix consensus sequences were used to BLAST against the mouse NCBI's RefSeq database. The highest scoring hit was used as the best hit (minimum e value ≤ 1e-10), and the corresponding GO terms were transferred from the mouse RefSeq sequences to the Affymetrix consensus sequence. Thus, 10,280 probesets on the GeneChip porcine genome array were assigned GO terms by Gene Ontology (www.geneontology.org). We further developed Perl scripts to create association files between interesting gene lists and corresponding GO terms for later use. Finally, specific GO-Slim, the full GO OBO flat file, and the association file of interesting genes, the map2slim script provided in go-dev, were used to count the number of times that a gene of interest was assigned a particular GO term. Fisher's exact test was used to estimate differences of each GO category in transcriptome and 848 differentially expressed genes ($p<0.01$, fold change>2, and $q<0.24$) and between genes from induced and repressed clusters.

NFκB pathway analysis

Pathway Studio 4.0.7 software (Ariadne Genomics Inc., Rockville, MD), which uses text-mining of scientific literature to identify interactions, was used to analyze and provide knowledge about molecular interaction networks. The software accepts human RefSeq ID as input, so the human RefSeq IDs were obtained by a blastall of the individual Affymetrix porcine consensus sequences against the entire RefSeq RNA and protein databases. An e value cutoff of 1e-10 was used for the RNA database and of 1e-5 for the protein database, along with a pattern match to the key word homo. The resulting file was parsed to obtain the human RefSeq IDs. Then, the human RefSeq IDs (for known porcine orthologs) of genes in the induced cluster and repressed cluster based on gene cluster analysis were used in this software to find common regulators (complex and protein only) of the gene list. Genes which had a direct connection with the NFκB complex were identified and were considered to be part of the pathway(s) controlled by NFκB.

Real-time quantitative PCR to analyze differentially expressed genes

Real-time quantitative PCR technology was used to verify the differential expression of 21 genes in early response stages (8, 24, and 48 hpi), as identified by the microarray. We also analyzed the expression of the TGM3 gene, which has not yet been annotated on the microarray. RPL32, a reference gene for high-abundance gene transcripts, was selected as a positive control. Total RNA was isolated from the MLN of the three noninfected pigs and the three infected pigs at each time point of 8, 24, and 48 hpi and reverse transcribed to cDNA using Superscript reverse transcriptase (Invitrogen, Carlsbad, CA) and oligo(dT) as previously described [23]. Real-time PCR was performed with 100 ng cDNA (RNA equivalent)/25 μl reaction/well using the Stratagene Brilliant kit (La Jolla, CA) on an ABI PRISM 7700 Sequence Detector System (Applied Biosystems). PCR conditions were 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min, then 4 °C. All probes and primers for real-time TaqMan PCR were designed as previously described [23]. The interpolated number (C_t) of cycles to reach a fixed threshold above the background noises was used to quantify amplification. The fold change in expression of the target gene was calculated as $2^{\Delta C_t}$, where ΔC_t is the difference between average C_t values for the control and infected pigs. Resulting Q-PCR data were analyzed by one-way ANOVA, on a gene-by-gene basis, that compared C_t values obtained from the noninfected and postinfection samples, using JMP 5.0 Software (SAS Inc.). Fisher's LSD post hoc test was applied to assess differences between groups of pigs at different time points postinfection. A value of $p \leq 0.05$ was considered statistically significant.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ygeno.2007.03.018.

References

[1] C.J. Thorns, Bacterial food-borne zoonoses, Rev. Sci. Tech. 19 (2000) 226–239.

[2] P.J. Fedorka-Cray, L.C. Kelley, T.J. Stabel, J.T. Gray, J.A. Laufer, Alternate routes of invasion may affect pathogenesis of *Salmonella typhimurium* in swine, Infect. Immun. 63 (1995) 2658–2664.

[3] R.L. Wood, R. Rose, Populations of *Salmonella typhimurium* in internal organs of experimentally infected carrier swine, Am. J. Vet. Res. 53 (1992) 653–658.

[4] B. Molla, et al., Multidrug resistance and distribution of *Salmonella* serovars in slaughtered pigs, J. Vet. Med. B. Infect. Dis. Vet. Public Health 53 (2006) 28–33.

[5] T. Humphrey, *Salmonella*, stress responses and food safety, Nat. Rev. Microbiol. 2 (2004) 504–509.

[6] J. Mermin, et al., Reptiles, amphibians, and human *Salmonella* infection: a population-based, case-control study, Clin. Infect. Dis. 38 Suppl 3 (2004) S253–S261.

[7] USDA, Ecology and epidemiology of *Salmonella* and other foodborne pathogens in livestock, Available online at http://www.%20nadc.ars.usda.gov/research/fs/epidemiology/ (verified 28 Oct. 2002). USDA, Washington, DC. 2001b.

[8] M.J. Wick, Living in the danger zone: innate immunity to *Salmonella*, Curr. Opin. Microbiol. 7 (2004) 51–57.

[9] P.R. Watson, S.M. Paulin, P.W. Jones, T.S. Wallis, Interaction of *Salmonella* serotypes with porcine macrophages in vitro does not correlate with virulence, Microbiology 146 (Pt. 7) (2000) 1639–1649.

[10] S.H. Zhao, et al., Gene expression profiling in *Salmonella* Choleraesuis-infected porcine lung using a long oligonucleotide microarray, Mamm. Genome 17 (2006) 777–789.

[11] K.A. Hyland, L. Kohrt, L. Vulchanova, M.P. Murtaugh, Mucosal innate immune response to intragastric infection by *Salmonella enterica* serovar Choleraesuis, Mol. Immunol. 43 (2006) 1890–1899.

[12] K.A. Skjolaas, T.E. Burkey, S.S. Dritz, J.E. Minton, Effects of *Salmonella enterica* serovar Typhimurium, or serovar Choleraesuis, *Lactobacillus reuteri* and *Bacillus licheniformis* on chemokine and cytokine expression in the swine jejunal epithelial cell line, IPEC-J2, Vet. Immunol. Immunopathol. (2006).

[13] J.J. Uthe, T.J. Stabel, S.H. Zhao, C.K. Tuggle, S.M. Bearson, Analysis of porcine differential gene expression following challenge with *Salmonella enterica* serovar Choleraesuis using suppression subtractive hybridization, Vet. Microbiol. 114 (2006) 60–71.

[14] C.M. Dvorak, et al., Gene discovery and expression profiling in porcine Peyer's patch, Vet. Immunol. Immunopathol. 105 (2005) 301–315.

[15] C. Tukel, et al., Neutrophil influx during non-typhoidal salmonellosis: who is in the driver's seat? FEMS Immunol. Med. Microbiol. 46 (2006) 320–329.

[16] T.A. Niewold, et al., The early transcriptional response of pig small intestinal mucosa to invasion by *Salmonella enterica* serovar typhimurium DT104, Mol. Immunol. 44 (2007) 1316–1322.

[17] I. Splichal, I. Trebichavsky, Y. Muneta, Y. Mori, Early cytokine response of gnotobiotic piglets to *Salmonella enterica* serotype typhimurium, Vet. Res. 33 (2002) 291–297.

[18] J.J. Uthe, et al., Porcine differential gene expression in response to *Salmonella enterica* serovars Choleraesuis and Typhimurium, Mol. Immunol. 44 (2007) 2900–2914.

[19] J. Schreiber, et al., Coordinated binding of NF-κB family members in the response of human cells to lipopolysaccharide, Proc. Natl. Acad. Sci. USA 103 (2006) 5899–5904.

[20] C.L. Afonso, et al., African swine fever virus multigene family 360 and 530 genes affect host interferon response, J. Virol. 78 (2004) 1858–1864.

[21] T.N. Ledger, et al., Development of a macroarray to specifically analyze immunological gene expression in swine, Clin. Diagn. Lab. Immunol. 11 (2004) 691–698.

[22] N. Baltes, G.F. Gerlach, Identification of genes transcribed by *Actinobacillus* pleuropneumoniae in necrotic porcine lung tissue by using selective capture of transcribed sequences, Infect. Immun. 72 (2004) 6711–6716.

[23] H.D. Dawson, et al., Localized multigene expression patterns support an evolving Th1/Th2-like paradigm in response to infections with *Toxoplasma gondii* and *Ascaris suum*, Infect. Immun. 73 (2005) 1116–1128.

[24] M. Ashburner, et al., Gene ontology: tool for the unification of biology. The Gene Ontology Consortium, Nat. Genet. 25 (2000) 25–29.

[25] R.G. Jenner, R.A. Young, Insights into host responses against pathogens from transcriptional profiling, Nat. Rev. Microbiol. 3 (2005) 281–294.

[26] L. Eckmann, M.F. Kagnoff, Cytokines in host defense against *Salmonella*, Microbes Infect. 3 (2001) 1191–1200.

[27] W.A. Meier, et al., Cytokines and synthetic double-stranded RNA augment the T helper 1 immune response of swine to porcine reproductive and respiratory syndrome virus, Vet. Immunol. Immunopathol. 102 (2004) 299–314.

[28] G.I. Solano-Aguilar, et al., Limited effect of recombinant porcine interleukin-12 on porcine lymphocytes due to a low level of IL-12 beta2 receptor, Vet. Immunol. Immunopathol. 89 (2002) 133–148.

[29] L. Eckmann, J.R. Smith, M.P. Housley, M.B. Dwinell, M.F. Kagnoff, Analysis by high density cDNA arrays of altered gene expression in human intestinal epithelial cells in response to infection with the invasive enteric bacteria *Salmonella*, J. Biol. Chem. 275 (2000) 14084–14094.

[30] B. John, et al., Role of IL-12-independent and IL-12-dependent pathways in regulating generation of the IFN-gamma component of T cell responses to *Salmonella typhimurium*, J. Immunol. 169 (2002) 2545–2552.

[31] A. Elhofy, K.L. Bost, Limited interleukin-18 response in *Salmonella*-infected murine macrophages and in *Salmonella*-infected mice, Infect. Immun. 67 (1999) 5021–5026.

[32] P. Mastroeni, et al., Interleukin 18 contributes to host resistance and gamma interferon production in mice infected with virulent *Salmonella* typhimurium, Infect. Immun. 67 (1999) 478–483.

[33] M.F. Roy, D. Malo, Genetic regulation of host responses to *Salmonella* infection in mice, Genes Immun. 3 (2002) 381–393.

[34] A. Spira, et al., Apoptosis genes in human alveolar macrophages infected with virulent or attenuated *Mycobacterium tuberculosis*: a pivotal role for tumor necrosis factor, Am. J. Respir. Cell Mol. Biol. 29 (2003) 545–551.

[35] M. Caraglia, et al., Alpha-interferon and its effects on signal transduction pathways, J. Cell Physiol. 202 (2005) 323–335.

[36] S.Y. Kim, Transglutaminase 2 in inflammation, Front. Biosci. 11 (2006) 3026–3035.

[37] A. Bouchon, J. Dietrich, M. Colonna, Cutting edge: inflammatory responses can be triggered by TREM-1, a novel receptor expressed on neutrophils and monocytes, J. Immunol. 164 (2000) 4991–4995.

[38] A. Bouchon, F. Facchetti, M.A. Weigand, M. Colonna, TREM-1 amplifies inflammation and is a crucial mediator of septic shock, Nature 410 (2001) 1103–1107.

[39] B. Ramanathan, J.E. Minton, C.R. Ross, F. Blecha, Cloning of porcine triggering receptor expressed on myeloid cells-1 (TREM-1) and its induction by lipopolysaccharide, peptidoglycan, and *Salmonella enterica* serovar Typhimurium infection, Dev. Comp. Immunol. 29 (2005) 1–7.

[40] C. Cheminay, A. Mohlenbrink, M. Hensel, Intracellular *Salmonella* inhibit antigen presentation by dendritic cells, J. Immunol. 174 (2005) 2892–2899.

[41] J.A. Tobar, P.A. Gonzalez, A.M. Kalergis, *Salmonella* escape from antigen presentation can be overcome by targeting bacteria to Fc gamma receptors on dendritic cells, J. Immunol. 173 (2004) 4058–4065.

[42] C.H. Lang, R.A. Frost, L.S. Jefferson, S.R. Kimball, T.C. Vary, Endotoxin-induced decrease in muscle protein synthesis is associated with changes in eIF2B, eIF4E, and IGF-I, Am. J. Physiol. Endocrinol. Metab. 278 (2000) E1133–E1143.

[43] R.A. Orellana, et al., Regulation of muscle protein synthesis in neonatal pigs during prolonged endotoxemia, Pediatr. Res. 55 (2004) 442–449.

[44] S.E. Calvano, et al., A network-based analysis of systemic inflammation in humans, Nature 437 (2005) 1032–1037.

[45] C.M. Tato, C.A. Hunter, Host-pathogen interactions: subversion and utilization of the NF-kappa B pathway during infection, Infect. Immun. 70 (2002) 3311–3317.

[46] A. Haraga, S.I. Miller, A *Salmonella enterica* serovar typhimurium translocated leucine-rich repeat effector protein inhibits NF-kappa B-dependent gene expression, Infect. Immun. 71 (2003) 4052–4058.

[47] S.C. Sun, P.A. Ganchi, D.W. Ballard, W.C. Greene, NF-kappa B controls expression of inhibitor I kappa B alpha: evidence for an inducible autoregulatory pathway, Science 259 (1993) 1912–1915.

[48] G. Totzke, et al., A novel member of the IkappaB family, human IkappaB-zeta, inhibits transactivation of p65 and its DNA binding, J. Biol. Chem. 281 (2006) 12645–12654.

[49] J.D. Storey, R. Tibshirani, Statistical significance for genome wide studies, Proc. Natl. Acad. Sci. USA 100 (2003) 9440–9445.

[50] M. Reich, K. Ohm, M. Angelo, P. Tamayo, J.P. Mesirov, GeneCluster 2.0: an advanced toolset for bioarray analysis, Bioinformatics 20 (2004) 1797–1798.